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Synergistic Effect of Sorafenib with Ionizing Radiation on Human Oral Cancer Cells

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Abstract. *Background/Aim: Although anticancer effects of sorafenib on renal, liver and colon carcinomas are well-known, its combination effect with ionizing radiation on oral squamous cell carcinoma (OSCC) is unclear. Herein human SAS cells, an OSCC cell line, were used in order to elucidate this combination effect. Materials and Methods: Both SAS and SAS/nuclear factor kappa-B-luciferase (SAS/NF-κB-luc2) cell lines were used in the study. Cell viability, NF-κB activation, and protein expression of NF-κB downstream effectors were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, NF-κB-luc2 reporter gene system, NF-κB/DNA binding activity and western blotting. Results: Sorafenib significantly increased radiation-induced cytotoxicity and apoptosis via both mitochondrial-dependent and independent pathways. In addition, NF-κB activity and downstream effector protein expression induced by radiation was suppressed by sorafenib in SAS/NF-κB-luc2 cells. Conclusion: Combination of sorafenib with radiation for the treatment of human OSCC shows a synergistic effect via suppression of radiation-induced NF-κB activity and its regulated downstream effector proteins.*

Human oral squamous cell carcinoma (OSCC) is a prevalent health problem worldwide, notably in south Asian countries. It was ranked the fifth cause of cancer mortality in Taiwan in 2012 (1). While several treatment regimens for human OSCC have been established, radiotherapy is still the standard adjuvant due to its ability to shrink tumor size (2, 3). However, most patients develop locoregional recurrence, which results in a poor survival rate (4). Therefore, development of effective therapy for the treatment of human OSCC is essential to improve patient survival.

Radioresistance has been reported to be correlated to several signaling pathways involving nuclear factor-kappaB (NF-κB) as the key molecule (5). The NF-κB signaling pathway is activated in several types of cancer, and its activation contributes to the malignant characteristics of cancer cells (6, 7). NF-κB downstream effector proteins such as cyclin D1, B-cell lymphoma 2 (BCL2), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), x-linked inhibitor of apoptosis protein (XIAP), matrix metalloproteinase 9 (MMP9), and cyclooxygenase-2 (COX2) contribute to radioresistance. These NF-κB–associated proteins promote tumor progression by enhancing cell proliferation, cell survival, invasion, metastasis, angiogenesis, and inflammation (8, 9). Irradiation activates the NF-κβ signaling pathway, leading to the transcription of these proteins and the development of radioresistance (10). NF-κB and its effector proteins have been shown to be correlated with poor radiotherapy response (11, 12). Therefore, the success of radiotherapy depends on finding a radiosensitive tumor target (13, 14).

The therapeutic efficacy of radiotherapy can be enhanced by inhibiting the NF-κB signaling pathway. By increasing cancer cell sensitivity, tumor growth can be better controlled. A radiosensitizer that sensitizes cancer cells and prevents normal tissue damage is ideal. Sorafenib, also known as BAY 43-9006 or Nexavar, is a multi-kinase inhibitor with activity against several pathways involving Rat-sarcoma (RAS)/mitogen-activated protein kinase/extracellular signal-regulated kinase (RAS/MEK/ERK), VEGF receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR) (15, 16). Sorafenib has been found to suppress 12-O-Tetradecanoylphorbol-13-acetate-induced VEGF and MMP9 expression *via* the ERK/NF-κB pathway in hepatocellular carcinoma (17). In our previous study, we

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found that sorafenib increased the cytotoxicity of radiation and inhibited protein expression downstream of NF-κB in colorectal cancer (18).

These results indicate that sorafenib may have the potential to moderate NF-κB activity and its effectors. However, whether sorafenib can sensitize oral squamous cell carcinoma (OSCC) to radiation through the inhibition of the NF-κB signaling pathway remains unclear. Herein, we investigated the efficacy of sorafenib combined with radiation on human OSCC SAS cells by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, western blotting, electrophoretic mobility shift assay (EMSA), transwell assay, DNA laddering assay, and a luciferase reporter gene assayed with bioluminescent imaging.

Materials and Methods

Cell culture and irradiation. The human oral cancer cell line SAS was kindly provided by professor Kuo-Wei Chang at the Department of Dentistry, National Yang-Ming University, Taipei, Taiwan. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% penicillin/streptomycin (Gibco®, Grand Island, NY, USA), and 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Rockford, IL, USA). G418 (500 μg/ml; Calbiochem, Darmstadt, Germany) was added to the medium to stabilize the SAS/NF-κB-*luc2* clone (see below). When cells reached 80% confluence, they were irradiated with an RS 2000 X-ray Biological Irradiator (Rad Source Technologies, Inc., Suwanee, GA, USA) at a dose rate of 1.03 Gy/min.

Plasmid transfection and stable clone selection. The NF-κB responsive element sequence was isolated from the pNF-κB-luc vector (Clontech, Mountain View, CA) with *Micrococcus luteus* (*Mlu*) I and *Haemophilus influenza* Rd (*Hind*) III, then blunted using the Klenow enzyme. The luc2 DNA sequence was isolated from pGL4-luc2 (Promega, Madison, WI, USA) with *Bacillus stearothermophilus* X (*BstX*) I and *Nocardia otitidiscaviarum* (*Not*) I and then blunted with the Klenow enzyme. The isolated *luc2* DNA sequence was inserted into pNF-κB-ires to form pNF-κB-ires-luc2. The resulting construction was named as pNF-κB-luc2 vector.

The transfection of SAS cells was performed using jetPEI™ (Polyplus Transfection, Strasbourg, France). SAS cells (2×10^6) were seeded in a 10-cm dish and allowed to grow for 24 h. The pNF-κB-luc2 vector (8 μg) and 16 μl of jetPEI™ solution were diluted with 500 μl and 484 μl of 145 mM NaCl. The mixture was mixed evenly, and then incubated at room temperature for 30 min. Then, the 1000-μl jetPEI™ /DNA mixture was added to the SAS cells and incubated at 37˚C for 24 h. Cells were then trypsinized and cultured with DMEM supplemented with 1 mg/ml G418, 1% penicillin/streptomycin, and 10% FBS for two weeks. The surviving clones were isolated and seeded into 96-well plates. The expression of Luc2 was assayed with bioluminescence imaging. The resulting cell clone was renamed as SAS/NF-κB-*luc2* cell line (16).

MTT assay. The MTT (Sigma-Aldrich, St. Louis, USA) assay was used to determine the cytotoxicity of sorafenib. SAS cells were seeded in 96-well plates (3×10^4 cells/well) for 24 h before treatment. Cells were then treated with different concentrations of sorafenib (0, 5, 10, 15, 20, and 25 μM) and radiation (10 Gy) in three separate groups: pre-treatment, concurrent treatment, and post-treatment for 24 h. In the pretreatment group, cells were treated with sorafenib for 3 h before irradiation. Concurrent treatment cells were treated with sorafenib (0-25 μM) and 10 Gy irradiation immediately. Lastly, the post-treatment group was treated with sorafenib (0-25 μM) 3 h after irradiation. Cells were washed with fresh medium, 100 μl of 5 mg/ml MTT solution was added, and then the cells were incubated for an additional 4 h. After removing the MTT solution, 100 μl of dimethyl sulfoxide (DMSO) was added for 5 min. The absorbance of the plate was then scanned with an ELISA reader (Power Wave X340; Bio-Tek Instrument, Inc., Winooski, VT, USA) at a wavelength of 570 nm.

NF-κB reporter gene assay. SAS/NF-κB-*luc2* cells were seeded into a 96-well plate at a cell density of 3×10^4/well and treated with 15 μM sorafenib, 10 μM ERK inhibitor (PD98059), and 5 nM NF-κB activation inhibitor [481406, 6-amino-4-(4-phenoxyphenylethylamino) quinazoline] (Thermo Scientific, Rockford, IL, USA) for 24 h. After 24 h, the medium was removed and 100 μl of 500 μM of D-luciferin was added to each well. Total photon signal was detected by a bioluminescence imaging system (Xenogen, Alameda, CA, USA). Regions of interest (ROIs) of the imaging were drawn in each well and quantified as photons per second by using Living Imaging software (Version 2.20; Xenogen).

Clonogenic formation assay. SAS cells (1×10^6) were seeded in 10 cm dishes incubated for 24 h, and then treated with or without 15 μM sorafenib. Cells were irradiated immediately with various irradiation doses (2-10 Gy) by using an X-ray irradiator (RS 2000; Rad Source Technologies Inc.) at a dose rate of 1.03 Gy/min, 80 cm source-to-cell distance (SCD), and field size of 30×30 cm^2. After irradiation, cells were then trypsinized, resuspended in fresh medium, and inoculated as a single cell in 10 cm diameter dishes. Two weeks later, the cell colonies were fixed with methanol: acetic acid (3:1) solution and stained with 2% crystal violet. The cell colony more than 50 cells was confirmed as survival. The ratio of surviving colonies to the number of plated cells with correction for plating efficiency was defined as the surviving fraction (SF). The radiosensitization effect of sorafenib on SAS cells was evaluated according to the formulas suggested by Valeriote and Lin (18) and Carpentier *et al*. (19), which are listed below.

Synergism, $SF_{R+S} < SF_R \times SF_s$; Additivity, $SF_{R+S}=SF_R \times SF_s$;
Sub-additivity, $SF_{R+S} > SF_R \times SF_s$, with $SF_{R+S} < SFR$ and $SF_{R+s} < SFs$;
Antagonism, $SF_{R+S} > SF_R$ or $SF_{R+S} > SF_S$,
where SF_R is the surviving fraction of SAS cells treated by radiation alone; SFS is the surviving fraction of SAS cells treated with 15 μM sorafenib alone ($SF_S=0.5$); SF_{R+S} is the surviving fraction of SAS cells treated by radiation plus15 μM sorafenib.

Extraction of sorafenib. The extraction of sorafenib from Nexavar tablet (Bayer Healthcare Co., Leverkusen, Germany) were conducted as mentioned previously (17).

EMSA. SAS cells were treated with 15 μM sorafenib, 10 Gy radiation, and a combination of both for 24 h. After treatment, nuclear proteins were extracted using the Nuclear Extraction Kit (Chemicon International, Temecula, CA, USA). The NF-κB binding activity was analyzed with the EMSA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). The NF-κB oligionucleotide sequence

was AGTTGAGGGGACTTTCCCAGGC. The non-labeled fragment sequence was GCCTGGGAAAGTCCCCTCAACT. Nuclear extracts were incubated with the biotin-labeled DNA probe for 20 min at 25˚C. A 5% polyacrylamide gel was used to separate DNA–protein complexes and free oligonucleotides, and then transferred to a nylon membrane. UV light was used to induce cross-linking. After incubation with streptavidin-horseradish peroxidase for 15 min, a chemiluminescent probe (ECL; Pierce, Thermo Scientific, Rockford, IL, USA) was added for detection by using a photographic film.

Transwell invasion assay. SAS cells were treated with 15 μM sorafenib, 10 Gy radiation, and a combination of both for 24 h. The cells (1×10^6) were suspended in serum-free medium in the transwell insert with 8-μm pores (Corning Costar, Corning, NY, USA). The bottom chamber was filled with medium containing 10% FBS. After incubation for 24 h, the transwell insert was washed with phosphate buffer saline (PBS) and fixed with methanol: acetone (3:1) for 15 min. The cells on the membrane were stained with hematoxylin for 10 min, and then air-dried. Images of the transwell insert were taken with a Leica DM IRB microscope (Deerfield, IL, USA). The number of cells was quantified by counting the number of cells from five different fields, averaging them, and then multiplying by the insert membrane growth area. The images were taken at a magnification of ×100.

Western blot analysis. Cells were seeded into 10-cm plates and treated with 15 μM sorafenib, 10 Gy radiation, and a combination of both for 24 h. After cells were harvested and lysed, the proteins (40 μg) were subjected to 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) and transferred onto polyvinylidene difluroide membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk, followed by the appropriate primary antibody (namely anti-BCL2, anti-VEGF, anti-MMP9, anti-XIAP, anti-cyclin-D1, anti-c-FLIP, anti-caspase-3, anti-caspase-8, anti-cytochrome c, anti-TNF-α, and COX2; Millipore) with gentle shaking overnight. Membranes were further incubated for 1 h at room temperature with secondary peroxidase-conjugated anti-rabbit or anti-mouse antibody. Protein expression was detected using an enhanced chemiluminescence system and the Image J software (National Institutes of Health, Bethesda, MA, USA) was used for quantification.

DNA fragmentation. SAS cells (1×10^6) were seeded in 6-well plates and treated with 15 μM sorafenib, 10 Gy radiation, and a combination of both for 24 h. The DNA fragments were extracted using a genomic DNA purification kit (Axygen Biosciences, Corning, MA, USA) following the manufacturer's instructions. The DNA fragmentation was assayed by 1.5% agarose gel electrophoresis in 0.5 M Tris/Borate/EDTA buffer.

Statistical analysis. All experiments were repeated three times independently. The statistical analysis was analyzed by the Student's *t*-test for determining significant difference. Data are represented as mean±standard error.

Results

Sorafenib suppressed tumor progression in SAS cells. Sorafenib increased the cytotoxicity of SAS cells in a dose-dependent manner (Figure 1A). The half-maximal inhibitory concentration (IC_{50}) of sorafenib were approximately 15 μM; therefore, 15 μM sorafenib were used for the remainder of this study. The expression of apoptotic proteins such as cleaved caspase-8, cleaved caspase-3, and cytochrome *c* was significantly increased (Figure 1B). Relative protein expression was measured and quantified. Our results indicate that sorafenib markedly inhibited MMP9, VEGF, cyclin-D1, COX2, TNF-α, BCL2, XIAP, and c-FLIP protein expression (Figure 1C). The quantification data suggest that sorafenib effectively suppresses NF-κB-mediated tumor progression-related protein expression in SAS cells. By observing the images of the transwell membrane, we found that sorafenib effectively reduced tumor cell migration in a time-dependent manner (Figure 1D).

Sorafenib suppressed NF-κB activation through ERK de-phosphorylation in SAS/NF-κB-luc2 cells. Our stable clone SAS/NF-κB-*luc2* was used to evaluate the relationship between sorafenib and NF-κB activity. Cells were treated with 15 μM sorafenib, ERK inhibitor (PD98059), and NF-κB activation inhibitor for 24 h, and the activation of NF-κB was investigated by a reporter gene assay. The NF-κB activation inhibitor was used as the postive control. Sorafenib markedly suppressed the activation of NF-κB compared to the control (Figure 2A). In addition, sorafenib inhibited ERK phosphorylation (Figure 2B).

Sorafenib sensitized SAS cells to radiation via inhibition of NF-κB–regulated gene products. Radiation significantly increased sorafenib-induced cytotocixity in SAS cells (Figure 3A). Cells were separated into three groups: cells pre-treated with sorafenib (15 μM) for 3 h and then subjected to radiation treatment (10 Gy), cells concurrently treated with sorafenib and radiation, and cells treated with sorafenib after radiation. After 24 h, the cell viability was analyzed with the MTT assay. No significant differences were observed between pre-treated cells and concurrently-treated cells; hence, for the rest of this study, the concurrent treatment was used. Concurrent treatment increased the cytotoxicity of radiation to SAS cells in a dose-dependent manner (Figure 3B). The combination treatment showed a synergistic cytotoxic effect compared with radiation alone (Table I). A DNA laddering assay was used to evaluate cell apoptosis. Figure 3C shows that the concurrent treatment clearly increased the fragmentation of DNA. We further evaluated the migration of SAS cells with a transwell assay. Irradiation enhanced the number of migratory cells, whereas sorafenib suppressed this phenomenon. The number of migratory cells was determined by counting five fields of each group and averaging them (Figure 3D). The NF-κB–associated gene products induced by radiation, such as MMP9, VEGF, cyclin-D1, TNF-α, and COX2, were suppressed by sorafenib (Figure 3E). Sorafenib suppressed the expression of anti-apoptotic proteins BCL2, XIAP, and c-FLIP, while it up-regulated the expression of pro-apoptotic proteins caspase-3, caspase-8, and cytochrome *c* (Figure 3F).

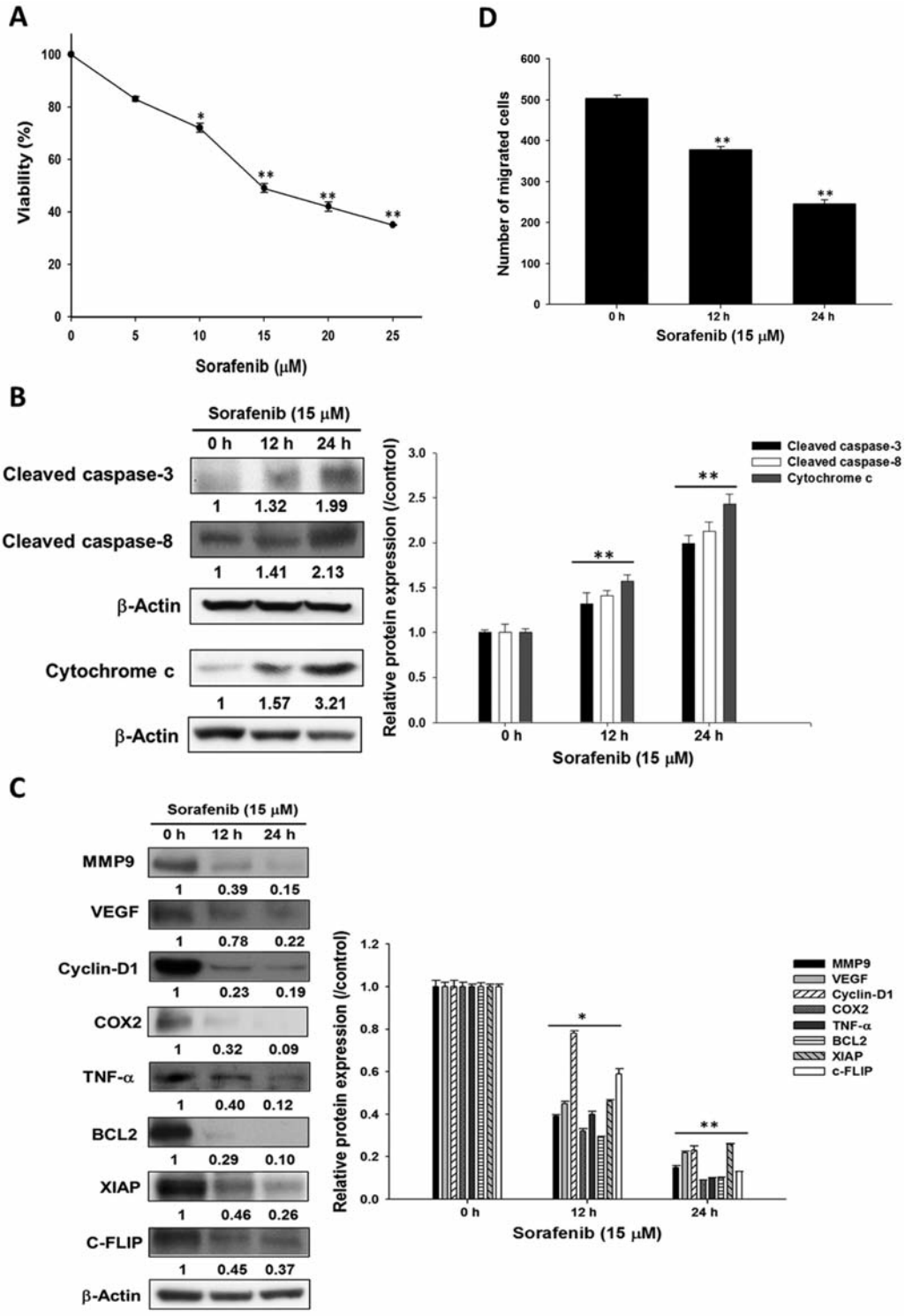

Figure 1. *Effect of sorafenib on SAS cells. A: The cytotoxicity of sorafenib was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. SAS cells were treated with different concentrations of sorafenib for 24 h. The half-maximal inhibitory concentration (IC_{50}) was 15 μM. B: Sorafenib significantly enhanced the expression of the apoptotic proteins cleaved caspase-3, cleaved caspase-8, and cytochrome c. β-Actin was used as the internal control. C: Nuclear factor-kappaB (NF-κB)-associated protein expression involved in invasion, angiogenesis, cell proliferation, inflammation, and anti-apoptosis was suppressed by sorafenib in a time-dependent manner. D: Cells were seeded into a transwell membrane for 24 h to evaluate the number of migratory cells. Sorafenib inhibited the number of migratory cells in a time-dependent manner. *p<0.05, **p<0.01 compared to sorafenib-treated cells for 0 h.*

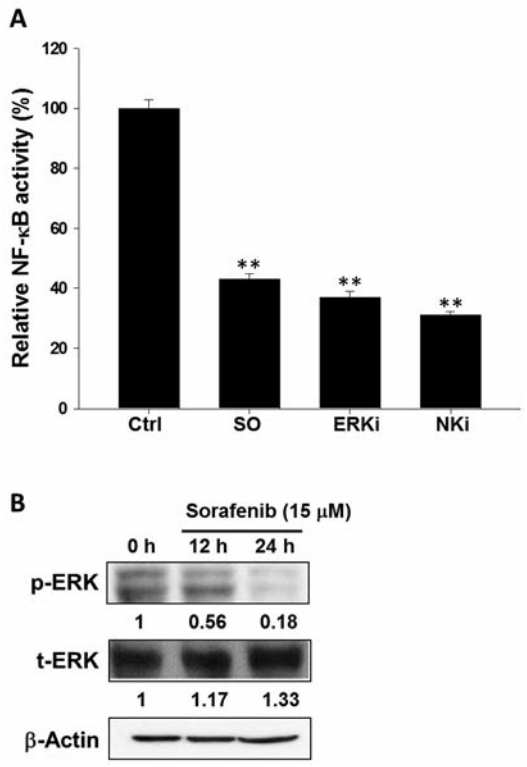

Figure 2. *Nuclear factor-kappaB (NF-κB) activity was inhibited by sorafenib (SO) via de-phosphorylation of extracellular signal-regulated kinase (ERK) in SAS/NF-κB-luc2 cells. A: SAS/NF-κB-luc2 cells were treated with sorafenib, ERK inhibitor (ERKi), and NF-κB activator inhibitor (NKi) for 24 h. NF-κB activation was inhibited by all three treatments. The quantification of the relative NF-κB activity by bioluminescence imaging was shown. B: Phosphorylated ERK was suppressed by sorafenib as shown by western blotting. The experiments were conducted three times. **p<0.01 compared with that of the DMSO-treated control (Ctrl). pERK: Phosphorylated extracellular signal-regulated kinase; tERK: total extracellular signal-regulated kinase.*

Sorafenib reduced radiation-induced NF-κB activity through ERK de-phosphorylation in SAS/NF-κB-luc2 cells. In Figure 4A, the activity of NF-κB induced by radiation was inhibited by sorafenib. Quantification data strongly indicate that sorafenib suppresses NF-κB activity induced by radiation. To investigate the NF-κB DNA-binding activity we used EMSA. NF-κB expression was enhanced by 1.46-fold by radiation and suppressed by 0.44-fold by sorafenib (Figure 4B). Sorafenib moderated the radiation-induced NF-κB activity *via* the ERK pathway (Figure 4C).

Discussion

Previous studies have shown that sorafenib inhibits the RAS/MEK/ERK pathway, as well as receptor tyrosine kinases such as VEGFR and PDGFR (6, 14). Chen *et al*. showed that the inhibition of the NF-κB signaling pathway effectively reduced invasion and metastasis of head and neck squamous cell carcinoma (21). In our previous study, we combined sorafenib with radiation for the treatment of human colon carcinoma and found that this treatment was effective (18). This finding suggested that sorafenib also suppresses NF-κB activity (Figure 2A) and expression of its downstream proteins (Figure 1C) *via* the ERK/NF-κB pathway in human oral cancer SAS cells (Figure 2B). In addition, sorafenib increased the expression of apoptotic proteins in SAS cells (Figure 1B) and reduced cell migration (Figure 1D). Therefore, we propose that using sorafenib as a NF-κB–targeting drug can sensitize SAS cells to radiation.

Table I. *The combination effect of 15 μM sorafenib on SAS cells was assayed and quantified according to the method of Valeriote and Carpeniter (19).*

Radiation (Gy)	SF_R	SF_{R+S}	$SF_R \times SF_S$
2	0.71	0.43 (Synergism)	0.56
4	0.66	0.21 (Synergism)	0.53
6	0.51	0.11 (Synergism)	0.40
8	0.31	0.05 (Synergism)	0.25
10	0.12	0.012 (Synergism)	0.096

SF_R: Surviving fraction of SAS cells treated by radiation alone; SF_S: surviving fraction of SAS cells treated with 15 μM sorafenib alone (SF_S=0.8); S_{FR+S}: surviving fraction of SAS cells treated by radiation plus 15 μM sorafenib.

Radiotherapy is known to inhibit cell proliferation, induce apoptosis, and activate several signal transduction pathways. Although radiation is the most commonly used treatment for cancer, its therapeutic efficacy decreases when cancer cells develop resistance to radiation. Radiation activates the NF-κB pathway and its downstream gene, thus contributing to the radioresistance of cancer cells (5, 10). Radiation induces NF-κB activity leading to an increase in the levels of the proteins involved in cell-cycle progression, invasion, and anti-apoptosis (Figure 3D). Radioresistance is a serious problem for patients with oral cancer treated with radiotherapy. Therefore, finding a treatment to control the negative effects of radiation may be the key to overcoming oral cancer. Huang *et al*. showed that the combination of radiotherapy or chemotherapy with biological agents to target the epithelial growth factor receptor is a potential treatment (3). In our study, we combined radiation with sorafenib to target NF-κB and evaluate the therapeutic efficacy of the combination therapy.

While it is already well-known that anti-apoptotic proteins such as BCL2 and XIAP are up-regulated by

radiation, we found that c-FLIPL was also enhanced. NF-κB is one of the several transcription factors that regulate c-FLIPL. Increased expression of c-FLIPL has been found in many types of cancer, one of which being head and neck squamous cell carcinoma (23, 24). Identifying a drug to target c-FLIP is difficult due to its structural similarity to caspase-8. Small-molecule drugs that can inhibit c-FLIP expression without inhibiting caspase-8 are required. Previous studies have evaluated several drugs that can reduce c-FLIP expression, allowing the cells to descend the apoptotic pathway (25, 26). In a previous study, Grant *et al*. evaluated the effect of sorafenib on human leukemia cells and found that it down-regulated cFLIPL (27). In the present study, our results indicate that sorafenib may also be a potential drug for targeting c-FLIPL in oral cancer (Figure 3D).

The NF-κB signal transduction pathway also plays a role in inflammation. When NF-κB is activated, several cytokines are transcribed, including the inflammatory cytokine TNF-α. Chronic inflammation has been shown to lead to tumor development due to alterations to the genome (28). COX2, another well-known inflammatory cytokine, is present in several malignant tumors, including oral squamous cell carcinoma (29). In the present study, we evaluated the protein expression of both TNF-α and COX2, which was enhanced by radiation. Sorafenib suppressed the expression of both these proteins by inhibiting the radiation-induced NF-κB activity (Figure 3E). In addition, our results show that sorafenib not only suppresses NF-κB DNA-binding activity (Figure 4B) and downstream proteins induced by irradiation (Figure 3E) but also enhances the apoptotic pathway (Figure 3F). In a cell viability assay, we found that concurrent treatment and sorafenib pre-treated with radiation can increase the cytotoxicity of SAS cells (Figure 3A). Our results indicate that sorafenib combined with radiation can inhibit radiation-induced NF-κB activity (Figure 4A). Moreover, sorafenib can suppress the migration ability (Figure 3C) and induce DNA fragmentation (Figure 3B) in SAS cells. This combined treatment not only suppresses the expression of proteins involved in anti-apoptosis, cell proliferation, cell migration, and cell survival, but also enhances the levels of apoptotic proteins in the human oral cancer cell line SAS.

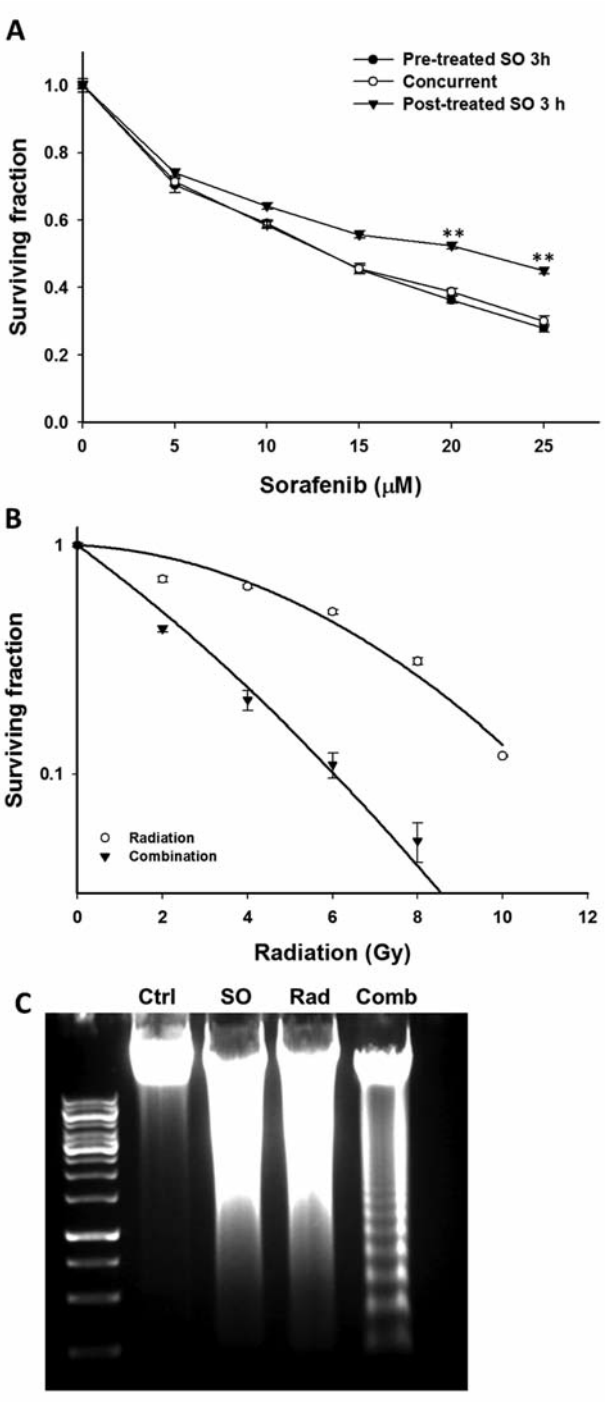

Figure 3. *continued*

Conclusion

Synergistic effect of sorafenib with radiation takes place *via* the suppression of NF-κB activity and expression of NF-κB-regulated downstream proteins. This combination strategy may have potential for human OSCC treatment in the clinic using sorafenib as a radiosensitizer to enhance the therapeutic effect.

Acknowledgements

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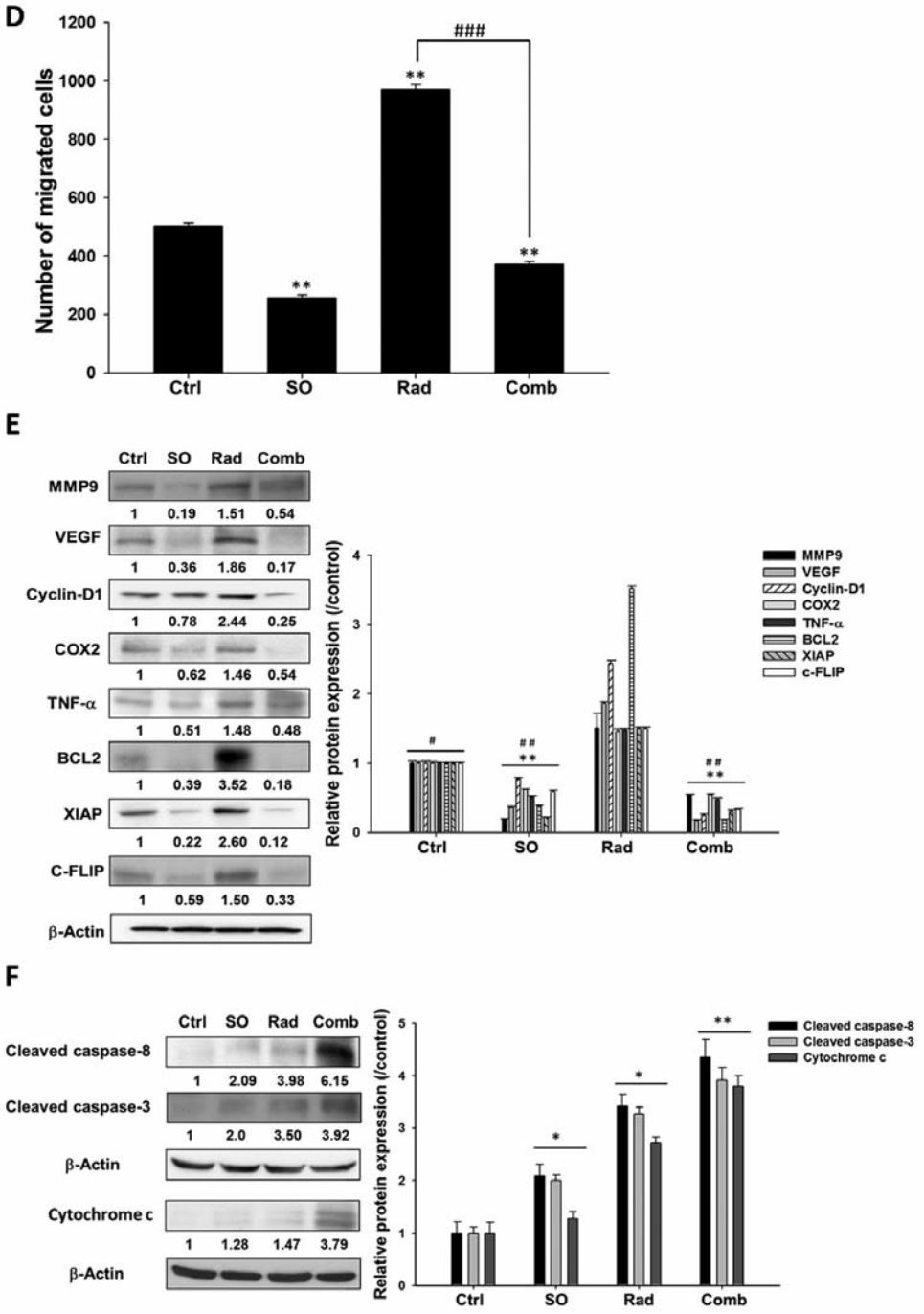

Figure 3. *The effect of sorafenib combined with radiation on tumor progression in SAS cells. A: SAS cells were pre-treated, concurrently treated, or post-treated with sorafenib and radiation. No significant difference was found between cell viability of the pre-treated and concurrently treated cells in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. B: Combination treatment significantly induced cytotoxicity of SAS cells according to the colony-formation assay. C: Sorafenib combined with radiation induced DNA fragmentation after 24 h of treatment in the DNA laddering assay. D: The combination treatment of sorafenib and radiation inhibited radiation-induced SAS cell migration. Five images for each field were selected to count the number of invading cells. E: Cells were irradiated and treated with sorafenib concurrently and incubated for 24 h. Protein expression was evaluated by western blotting. Sorafenib suppressed radiation-induced expression of NF-κB-associated proteins involved in tumor progression, Matrix metalloprotein 9 (MMP9), vascular endothelial growth factor (VEGF), cyclin D1, cyclooxygenase 2 (COX2), tumor-necrosis factor alpha (TNF-α), B-cell lymphoma 2 (BCL2), X-linked inhibitor of apoptosis protein (XIAP), and cellular FLICE-like inhibitory protein (c-FLIP). F: Apoptotic protein expression in SAS cells was markedly increased by the combined treatment of sorafenib and radiation. *$p<0.05$, **$p<0.01$ as compared with the DMSO-treated control (Ctrl). $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ as compared with group treated with radiation alone. SO: Sorafenib; Rad: radiation; Comb: combination.*

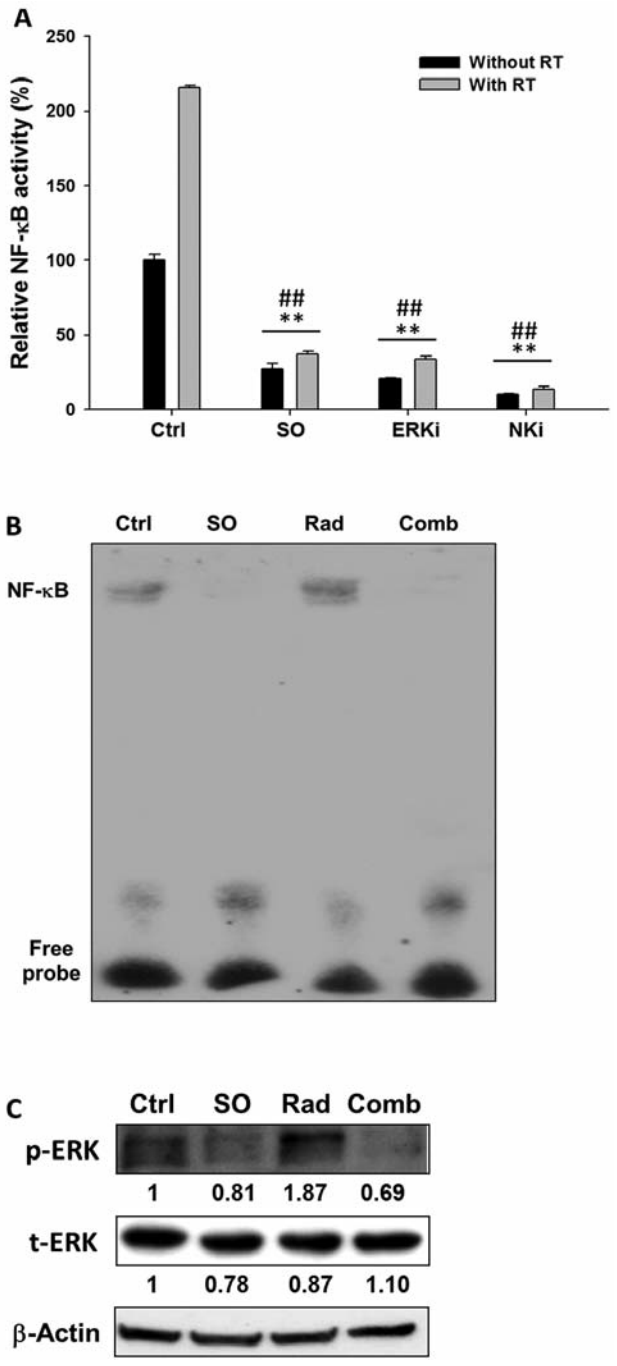

Figure 4. *Sorafenib suppressed radiation-induced nuclear factor kappa-B (NF-κB) activity in SAS cells. A: The activation of NF-κB was evaluated by bioluminescent imaging. NF-κB activation was suppressed by sorafenib (SO), ERK inhibitor (ERKi), and NF-κB activator inhibitor (NKi). Radiation enhanced NF-κB activation, while the combined treatment suppressed it. B: Radiation-induced NF-κB DNA-binding activity in SAS cells was inhibited by sorafenib. C: The combined treatment with sorafenib reduced phosphorylated extracellular signal-regulated kinase (p-ERK) expression induced by radiation. All experiments were conducted three times. **p<0.01 compared to the DMSO-treated control (Ctrl). ##p<0.01 compared to group treated with radiation-alone.*

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